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## (54) Protein, DNA and use thereof.

(57) Disclosed are (1) a Xenopus laevis bone morphogenetic protein (BMP), (2) a DNA comprising a DNA segment coding for a Xenopus laevis BMP, (3) a transformant bearing a DNA comprising a DNA segment coding for a Xenopus laevis BMP and (4) a method for preparing the Xenopus laevis BMP which comprises culturing the described in (3), producing and accumulating the protein in a culture, and collecting the protein thus obtained. Cells transinfected or transformed with the DNA allow large amounts of the Xenopus laevis BMP mature peptides to be produced, which causes the advantageous production of the peptides, which promote the synthesis of proteoglycan and can also be utilized for analysis of the mechanism of organism, particularly human bone-cartilage morphogenetic reaction, and as therapeutic agents for osteoporosis.

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# PROTEIN, DNA AND USE THEREOF

## BACKGROUND OF THE INVENTION

The present invention relates to a DNA containing a DNA segment coding for a Xenopus laevis bone morphogenetic protein analogous to a bone morphogenetic protein (hereinafter referred to as BMP), a precursor protein (or a precursor polypeptide) and a mature protein (or a mature polypeptide) of the Xenopus laevis BMP, and a method for preparing the precursor protein and the mature protein.

In this specification, the term "precursor protein" includes a protein which includes an amino acid sequence of a mature peptide Xenopus Laevis BMP and has all or a portion of an amino acid sequence coded with a Xenopus laevis BMP DNA segment at the N-terminus, the C-terminus or both termini thereof.

Recently, it has been revealed that transforming growth factor-beta (TGF-beta, TGF-$\beta$) having a bone morphogenetic activity not only controls cell proliferation, but also has various biological activities such as control of cell differentiation. In particular, the bone morphogenesis-promoting activity of TGF-$\beta$ has been noted, and attempts have been made to use TGF for treatment of fractures and osteoporosis, making use of the cartilage-bone induction activity thereof [M. Noda et al., J. Endocrinology 124 , 2991-2994 (1989); M. E. Joyce et al., J. Bone Mineral Res . 4, S-259 (1989); and S. M. Seyedin et al., J. Biol. Chem. 281 , 5693-5695 (1986)]. More recently, however, four kinds of bone morphogenetic proteins (BMPs) which are different from one another in molecular structure have been identified as a factor promoting morphogenesis of bones and cartilages. Of these four kinds, human BMP-1, human BMP-2A, human BMP-2B and human BMP-3 are novel peptides, though they are very similar in structure to TGF-$\beta$, and there has been a report that they induce morphogenesis of bones and cartilages when subcutaneously or intramuscularly implanted in animals [J. M. Wozney et al., Science 242 , 1528-1534 (1989)].

The above peptides having bone morphogenetic activity are isolated and purified from bones in which the peptides are considered to be localized, or from human osteosarcoma cells (U2-OS) which are thought to produce the peptides. However, such a method has problems because the procedure is complicated and the desired peptides are obtained only in small amounts.

## SUMMARY OF THE INVENTION

Important contributions will be made to future studies and medical treatment, if a similar peptide having the bone morphogenetic activity can be collected from Xenopus laevis and further prepared by recombinant technique. As a result, the following information was obtained, thus arriving at the present invention.

Namely, the present inventors first succeeded in cloning five kinds of DNA coding for BMP-2A and related DNAs ( Xenopus laevis BMPs) and subsequently three kinds of complementary DNAs, eight kinds of DNAs in total, by using a complementary DNA of a rat inhibin $\beta$A chain equally belonging to the TGF-$\beta$ family as a probe. Further, the present inventors identified portions of the bases of the DNAs, clarified the amino acid sequences (see formulae (I), (II), (III), (IV) and (V) of Fig. 3 and formulae (VI), (VII) and (VIII) of Fig. 4) of the Xenopus laevis BMPs (referred to as B9, M3, C4, A4, A5, Xbr22, Xbr23 and Xbr41), and succeeded in pioneering their mass production by recombinant technique.

In accordance with the present invention, there are provided (1) a Xenopus laevis BMP, (2) a DNA comprising a DNA segment coding for the Xenopus laevis BMP, (3) a transformant bearing the DNA containing the DNA segment coding for the Xenopus laevis BMP and (4) a method for preparing the Xenopus laevis BMP which comprises culturing the transformant described in (3), producing and accumulating a protein in a culture and collecting the protein thus obtained.

## BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows simplified restriction enzyme maps of DNA sequences containing Xenopus laevis BMP precursors or mature peptide DNA segments;

Figs. 2(1) to 2(8) show nucleotide sequences of the DNA segments of Xenopus laevis BMPs, $B_9$, M3, C4, A4, A5, BMP-2A, BMP-2B and Vgr-1, respectively, and the amino acid sequences deduced therefrom;

Fig. 3 shows amino acid sequences of the Xenopus laevis BMPs deduced from the nucleotide sequences of the DNA segments shown in Figs. 2(1) to 2(5), comparing them with the amino acid sequences of known proteins having a bone morphogenetic activity; and

Fig. 4 shows amino acid sequences of the Xenopus laevis BMPs deduced from the nucleotide sequences of the cDNA segments shown in Figs. 2(6) to 2(8).

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

The mature Xenopus laevis BMP of C4, one of the Xenopus laevis BMPs, of the present invention, which has a relationship to TGF-$\beta$ and is a peptide consisting of 98 or 114 amino acid residues, has an amino acid sequence represented by Nos. 6 to 119 or Nos. 22 to 119 of formula (III) shown in Fig. 3. The molecular weight thereof is calculated at about 25,000, excepting sugar chains, when a dimer is formed.

The amino acid sequence of this peptide is different from that reported by Wozney et al. in 3 or 4 amino acid residues per molecule.

Fig. 3 shows amino acid sequences of five kinds of novel Xenopus laevis BMPs obtained in the present invention, comparing them with the amino acid sequences of known proteins having a bone morphogenetic activity. In these amino acid sequences, the same amino acid residue as with $\beta$A is represented by ".", and an amino acid residue different from that of $\beta$A is represented by one letter symbol based on $\beta$A. CONSENSUS shown in Fig. 3 indicates amino acid residues common to all the BMPs shown in Fig. 3. The illustration of CONSENSUS results in introduction of gaps "-" in the formulae in Fig. 3. Accordingly, the number representing the precursor and mature protein portions is counted excluding these lacking portions.

Fig. 4 shows amino acid sequences of three kinds of novel Xenopus laevis BMPs deduced from cDNAS, subsequently discovered by the present inventors.

For DNA sequences, the DNA segments coding for the Xenopus laevis BMPs of the present invention correspond to the nucleotide sequences of formulae (1) to (8) (corresponding to B9, M3, C4, A4, A5, Xbr22, Xbr23 and Xbr4l, respectively) shown in Fig. 2 or are portions thereof. Any functional portion can be used so long as bone morphogenetic activity is not lost. Wozney et al. reports the amino acid sequences, but does not elucidate the nucleotide sequences. As used herein the term correspond permits conservative additions, deletions and substitutions. Preferably, the DNA segments coding for the BMPs of the present invention have the nucleotide sequences of formulae (1) to (8).

With respect to the portion relating to the mature BMPs [the amino acid sequence represented by Nos. 15 to 130 of formula (I) shown in Fig. 3, the amino acid sequence represented by Nos. 14 to 127 of formula (II) shown in Fig. 3, the amino acid sequence represented by Nos. 6 to 119 or

Nos. 22 to 119 of formula (III) shown in Fig. 3, the amino acid sequence represented by Nos. 6 to 63 of formula (IV) shown in Fig. 3, the amino acid sequence represented by Nos. 6 to 65 of formula (V) shown in Fig. 3, the amino acid sequence represented by Nos. 282 to 398 or Nos. 298 to 398 of formula (VI) shown in Fig. 4, the amino acid sequence represented by Nos. 288 to 401 or Nos. 304 to 401 of formula (VII) shown in Fig. 4, or the amino acid sequence represented by Nos. 328 to 426 of formula (VIII) shown in Fig. 4], the DNA sequences of the present invention differ from the DNA sequence of TGF-$\beta$, and therefore are novel.

As the DNA sequences coding for the BMP mature peptides of the present invention, any DNA sequences may be used as long as they contain nucleotide sequences coding for the amino acid sequences of the BMP mature peptides. For example, DNA sequences corresponding to the nucleotide sequences represented by formulae (1) to (8) or portions thereof are preferably used. More preferably the DNA sequences contain the nucleotide sequences represented by formulae (1) to (8).

The nucleotide sequences represented by formulae (1) to (8) are the Xenopus laevis BMP DNA sequences obtained in the present invention. Examples of the nucleotides coding for the Xenopus laevis BMP amino acid sequences represented by formulae (I) to (VIII) include Nos. 693 to 1040 of formula (1), Nos. 134 to 475 of formula (2), Nos. 435 to 728 of formula (3), Nos. 183 to 356 of formula (4), Nos. 149 to 328 of formula (5), Nos. 249 to 1442 of formula (6), Nos. 104 to 1306 of formula (7) and Nos. 86 to 1363 of formula (8).

An expression vector having the DNA sequence containing the nucleotide sequence coding for the BMP of the present invention can be prepared, for example, by the following process:

(a) Messenger RNA (MRNA) is isolated from BMP-producing cells.

(b) Single stranded complementary DNA (cDNA) is synthesized from the mRNA, followed by synthesis of double stranded DNA.

(c) The complementary DNA is introduced in a cloning vector such as a phage or a plasmid.

(d) Host cells are transformed with the recombinant phage or plasmid thus obtained.

(e) After cultivation of the transformant thus obtained, the plasmid or the phage containing the desired DNA is isolated from the transformant by an appropriate method such as hybridization with a DNA probe coding for a portion of the BMP or immunoassay using an anti-BMP antibody.

(f) The desired cloned DNA sequence is cut out from the recombinant DNA.

(g) The cloned DNA sequence or a portion thereof is ligated downstream from a promoter in the expression vector.

The mRNAs coding for the BMPs can be obtained from various BMP-producing cells such as ROS cells.

Methods for preparing the mRNAs from the BMP-producing cells include the guanidine thiocyanate method [J. M. Chirgwin et al., Bio-chemistry 18 , 5294 (1979)].

Using the MRNA thus obtained as a template, cDNA is synthesized by use of reverse transcriptase, for example, in accordance with the method of H. Okayama et al. [ Molecular and Cellular Biology 2 , 161 (1979); ibid. 3 , 280 (1983)]. The cDNA thus obtained is introduced into the plasmid.

The plasmids into which the cDNA is introduced include, for example, pBR322 [ Gene 2 , 95 (1977)], pBR325 [ Gene 4 , 121 (1978)], pUC12 [ Gene 19 , 259 (1982)] and pUC13 [ Gene 19 , 259 (1982)], each derived from Escherichia coli , and pUB110 derived from Bacillus subtilis [ Biochemical and Biophysical Research Communication 112 , 678 (1983)]. However, any other plasmids can be used as long as they are replicable and growable in the host cells. Examples of the phage vectors into which the cDNA may be introduced include λgtII [R. Young and R. Davis, Proc. Natl. Acad. Sci. U.S.A. 80 , 1194 (1983)]. However, any other phage vectors can be used as long as they are growable in the host cells.

Methods for introducing the cDNA in the plasmid include, for example, the method described in T. Maniatis et al., Molecular Cloning , Cold Spring Harbor Laboratory, p.239 (1982). Methods for introducing the cDNA in the phage vector include, for example, the method of T. V. Hyunh et al. [ DNA Cloning, A Practical Approach 1 , 49 (1985)].

The plasmid thus obtained is introduced into the appropriate host cell such as Escherichia and Bacillus

Examples of Escherichia described above include Escherichia coli K12DH1 [ Proc. Natl. Acad. Sci. U.S.A. 60 , 160 (1968)], M103 [ Nucleic Acids Research 9 , 309 (1981)], JA221 [ Journal of Molecular Biology 120 , 517 (1978)], HB101 [ Journal of Molecular Biology 41 , 459 (1969)] and C600 [ Genetics 39 , 440 (1954)].

Examples of Bacillus described above include Bacillus subtilis MI114 [ Gene 24 , 255 (1983)] and 207-21 [ Journal of Biochemistry 95 , 87 (1984)].

Methods for transforming the host cell with the plasmid include, for example, the calcium chloride method or the calcium chloride/rubidium chloride method described in T. Maniatis et al., Molecular Cloning , Cold Spring harbor Laboratory, p.249 (1982).

When the phage vector is used, for example, the phage vector can be transduced into multiplied Escherichia coli , using the in vitro packaging method.

Xenopus laevis cDNA libraries containing Xenopus laevis BMP cDNA can be obtained by numerous techniques well known in the art including purchasing them from the market, though obtainable by the methods described above. For example, the cDNA library of Xenopus laevis is available from Clontech Laboratories, Inc., U.S.A.

Methods for cloning the Xenopus laevis BMP DNA from the Xenopus laevis DNA library include, for example, the plaque hybridization method using phage vector λcharon 28A and rat inhibin (activin) βA cDNA as probes [T. Maniatis et al., Molecular Cloning, Cold Spring Harbor Laboratory, (1982)].

The Xenopus laevis BMP DNA thus cloned is subcloned in plasmids such as pBR322, pUC12, pUC13, pUC19, pUC118 and pUC119 to obtain the Xenopus laevis BMP DNA, if necessary.

The nucleotide sequence of the DNA sequence thus obtained is determined, for example, by the Maxam-Gilbert method [A. M. Maxam and W. Gilbert, Proc. Natl. Acad. Sci. U.S.A. 74 , 560 (1977)] or the dideoxy method [J. Messing et al., Nucleic Acids Research 9 , 309 (1981)], and the existence of the Xenopus laevis BMP DNA is confirmed in comparison with the known amino acid sequence.

As described above, the DNA sequence [ Xenopus laevis BMP DNAs represented by formulae (1) to (8)] coding for the Xenopus laevis BMPs are obtained.

Fig. 1 shows the restriction enzyme fragment maps of the DNA sequences containing the DNA segments coding for the Xenopus laevis BMPs obtained in Example 1 described below. Fig. 2 shows the nucleotide sequences represented by formulae (1) to (8) of the DNA sequences as determined by the dideoxy method, and Figs. 3 and 4 show the amino acid sequences represented by formulae (I) to (V) and formulae (VI) to (VIII), respectively, which were ascertained form the above nucleotide sequences.

The DNA sequence coding for the Xenopus laevis BMP cloned as described above can be used as it is, or after digestion with a restriction enzyme if desired, depending on the intended use.

A region intended to be expressed is cut out from the cloned DNA and ligated downstream from the promoter in a vehicle (vector) suitable for expression, whereby the expression vector can be obtained.

The DNA sequence has ATG as a translation initiating codon at the 5′-terminus thereof and may have TAA, TGA or TAG as a translation terminating codon at the 3′-terminus. The translation initiating codon and translation terminating codon may be added by use of an appropriate synthetic DNA adaptor. The promoter is further ligated in the upstream thereof for the purpose of expressing the DNA sequence.

Examples of the vectors include the above plasmids derived from E. coli such as pBR322, pBR325, pUC12 and pUC13, the plasmide derived from B. subtilis such as pUB110, pTP5 and pC194, plasmids derived from yeast such as pSH19 and pSH15, bacteriophage such as λphage, and animal viruses such as retroviruses and vaccinia viruses.

As the promoters used in the present invention, any promoters are appropriate as long as they are suitable for expression in the host cells selected for the gene expression.

When the host cell used for transformation is Escherichia , it is preferable that a trp promoter, a lac promoter, a recA promoter, a λPL promoter, a lpp promoter, etc. are used. When the host cell is Bacillus , it is preferable that a PHO5 promoter, a PGK promoter, a GAP promoter, an ADH promoter, etc. are used. In particular, it is preferable that the host cell is Escherichia and the promoter is the trp promoter or the λPL promoter.

When the host cell is an animal cell, an SV-40 derived promoter, a retrovirus promoter, a metallothionein promoter, a heat shock promoter, etc. are each usable.

An enhancer, a certain DNA sequence important for promoter activity in a cell, is also effectively used for expression.

By using the vector containing the DNA sequence coding for the Xenopus laevis BMP mature peptide thus constructed, the transformant is prepared.

The host cell include, for example, Escherichia , Bacillus, yeast and animal cells.

Specific examples of the above Escherichia and Bacillus include strains similar to those described above.

Examples of the above yeast include Saccharomyces cerevisiae AH22, AH22R⁻, NA87-11A and DKD-5D.

Examples of animal cells include monkey cell COS-7, Vero, Chinese hamster cell (CHO), mouse L cell and human FL cell.

The transformation of the above Escherichia is carried out, for example, according to the method described in Proc. Natl. Acad. Sci. U.S.A. 69 , 2110 (1972) or Gene 17 , 107 (1982).

The transformation of the above Bacillus is conducted, for example, according to the method described in Molecular & General Genetics 168 , 111 (1979).

The transformation of the yeast is carried out, for example, according to the method described in Proc. Natl. Acad. Sci. U.S.A. 75 , 1929 (1978).

The transformation of the animal cells is carried out, for example, according to the method described in Virology 52 , 456 (1973).

Thus, there is obtained the transformant transformed with the expression vector containing the DNA sequence coding for the Xenopus laevis BMP mature peptide.

When bacterial transformants are cultured, a liquid medium is particularly suitable as a medium used for culture. Carbon sources, nitrogen sources, inorganic compounds and others necessary for growth of the transformant are contained therein. Examples of the carbon sources include glucose, dextrin, soluble starch and sucrose. Examples of the nitrogen sources include inorganic or organic materials such as ammonium salts, nitrates, corn steep liquor, peptone, casein, meat extracts, soybean meal and potato extract solution. The inorganic compounds include, for example, calcium chloride, sodium dihydrogenphosphate and magnesium chloride. Yeast extract, vitamins, growth promoting factors and so on may be further added thereto.

The pH of the medium is preferably about 5 to 8.

As the medium used for cultivation of Escherichia , there is preferred, for example, M9 medium containing glucose and Casamino Acids (Miller, Journal of Experiments in Molecular Genetics 431-433 , Cold Spring Harbor Laboratory, New York, 1972). In order to make the promoter act efficiently, a drug such as 3β-indolylacrylic acid may be added thereto if necessary.

When the host cell is Escherichia , the cultivation is usually carried out at about 15 to 43° C for about 3 to 24 hours, with aeration or agitation if necessary.

When the host cell is Bacillus , the cultivation is usually carried out at about 30 to 40° C for about 6 to 24 hours, with aeration or agitation if necessary.

When yeast transformants are cultured, there is used, for example, Burkholder minimum medium [K. L. Bostian et al., Proc. Natl. Acad. Sci. U.S.A. 77 , 4505 (1980)] as the medium. The pH of the medium is preferably adjusted to about 5 to 8. The cultivation is usually carried out at about 20 to 35° C for about 24

to 72 hours, with aeration or agitation if necessary.

When animal cell transformants are cultured, examples of the media include MEM medium containing about 5 to 20% fetal calf serum [ Science 122 , 501 (1952)], DMEM medium [ Virology 8 , 396 (1959)], RPMI1640 medium [ Journal of the American Medical Association 199 , 519 (1967)] and 199 medium [ Proceeding of the Society for the Biological Medicine 73 , 1 (1950)]. The pH is preferably about 6 to 8. The cultivation is usually carried out at about 30 to 40 °C for about 15 to 60 hours, with aeration or agitation if necessary.

The above Xenopus laevis BMP mature peptide can be isolated and purified from the culture described above, for example, by the following method.

When the Xenopus laevis BMP mature peptide is to be extracted from the cultured cells, the cells are collected by methods known in the art after cultivation. Then, the collected cells are suspended in an appropriate buffer solution and disrupted by ultrasonic treatment, lysozyme and /or freeze-thawing. Thereafter, a crude extracted solution of the Xenopus laevis BMP mature peptide is obtained by centrifugation or filtration. The buffer solution may contain a protein denaturant such as urea or guanidine hydrochloride, or a surface-active agent such as Triton X-100.

When the Xenopus laevis BMP precursor protein or mature peptide is secreted in the culture solution, a supernatant is separated from the cells by methods known in the art after the conclusion of cultivation, and then collected.

The separation and purification of the Xenopus laevis BMP precursor protein or mature peptide contained in the culture supernatant or the extracted solution thus obtained can be performed by an appropriate combination of known separating and purifying methods. The known separating and purifying methods include methods utilizing solubility such as salt precipitation and solvent precipitation, methods mainly utilizing a difference in molecular weight such as dialysis, ultrafiltration, gel filtration and SDS-polyacrylamide gel electrophoresis, methods utilizing a difference in electric charge such as ion-exchange column chromatography, methods utilizing specific affinity such as affinity chromatography, methods utilizing a difference in hydrophobicity such as reverse phase high performance liquid chromatography and methods utilizing a difference in isoelectric point such as isoelectro-focusing electrophoresis. Methods using an antibody to a fused protein expressed by fusing BMP complementary DNA or DNA with E. coli -derived DNA lacZ can also be used.

Illustrative examples of the methods for expressing the BMP in the present invention include methods in which genes are introduced into CHO cells to produce the BMP in large amounts as described in Wang et al., Proc. Natl. Acad. Sci. U.S.A. 807 , 2220-2224 (1990).

The activity of the Xenopus laevis BMP precursor protein or mature peptide thus formed can be measured by an enzyme immunoassay using a specific antibody. If the products have a bone morphogenetic activity, this activity may also be measured as an index.

The cells, such as animal cells or E. coli , transinfected or transformed with the DNA sequences of the present invention allow large amounts of the Xenopus laevis BMP mature peptides to be produced. Hence, the production of these peptides can be advantageously achieved.

It has become clear that the Xenopus laevis BMP mature peptides prepared here promote the synthesis of proteoglycan which is a main component of a cartilage matrix, and the peptides can also be utilized for analysis of the mechanism of organism, particularly human bone-cartilage morphogenetic reaction, and as therapeutic agents for fracture or osteoporosis.

In such instances one would administer an effective amount of the protein to a mammal. An effective amount is the amount of protein needed to promote the synthesis of proteoglycan in cartilage cells. Typically, this ranges from 0.001 to 35 µg per kg/body weight. The precise amount for a particular purpose can readily be determined empirically by the person of ordinayl skill in the art based upon the present disclosure.

When one uses the protein for therapeutic purpose care is taken to purify it and render it substantially free of bacterica and pyrogens. This can be done by standard methods.

When the BMPs are used as therapeutic agents for fracture or osteoporosis, they can be administered parenterally in the forms of solutions, injections and ointments, solely or in combination with pharmaceutically acceptable additional components, such as vehicles, binders, dispersants, plasticizers or diluents.

The preferable administration forms include (1) administration of the agent to cutis surface near a diseased part, (2) injection of the agent into a diseased part, (3) discission of a diseased part followed by direct administration of the agent thereto. The preferable dose in fractue therapy for adult people is 0.1 to 2000 µg more, preferably 20 to 400 µg for adult people once a day. The preferable dose in osteoporosis for adult people is 0.1 to 200 µg once a day, for about one to 30 days. The concentration of the therapeutic agent is, preferably, 0.001 to 0.2% in the form of a solution, 0.001 to 0.2% in the form of an injections, and

0.0001 to 0.2% in the form of an ointment.

There have been described above in detail the cloning of the DNA sequences coding for the Xenopus laevis BMPs, the preparation of the expression vectors for the Xenopus laevis BMP mature peptides, the production of the transformants by using the transformants and their utility.

When nucleotides, amino acids and so on are indicated by the abbreviations in this specification and drawings, the abbreviations adopted by IUPAC-IUB Commission on Biochemical Nomenclature or commonly used in the art are employed. For example, the following abbreviations are used. When the amino acids are capable of existing as optical isomer, the L-forms are represented unless otherwise specified.

DNA : Deoxyribonucleic acid
cDNA : Complementary deoxyribonucleic acid
A : Adenine
T : Thymine
G : Guanine
C : Cytosine
RNA : Ribonucleic acid
mRNA : Messenger ribonucleic acid
dATP : Deoxyadenosine triphosphate
dTTP : Deoxythymidine triphosphate
dGTP : Deoxyguanosine triphosphate
dCTP : Deoxycytidine triphosphate
ATP : Adenosine triphosphate
EDTA : Ethylenediaminetetraacetic acid
SDS : Sodium dodecyl sulfate
Gly or G : Glycine
Ala or A : Alanine
Val or V : Valine
Leu or L : Leucine
Ile or I : Isoleucine
Ser or S : Serine
Thr or T : Threonine
Cys or C : Cysteine
Met or M : Methionine
Glu or E : Glutamic acid
Asp or D : Aspartic acid
Lys or K : Lysine
Arg or R : Arginine
His or H : Histidine
Phe or F : Phenylalanine
Tyr or Y : Tyrosine
Trp or W : Tryptophan
Pro of P : Proline
Asn or N : Asparagine
Gln or Q : Glutamine

With respect to the Xenopus laevis BMP mature peptides of the present invention, a portion of the amino acid sequence may be modified, namely there may be addition, elimination or substitution with other amino acids as long as the bone morphogenetic activity is not lost.

The present invention will hereinafter be described in detail with the following Examples. It is understood of course that these Examples are not intended to limit the scope of the invention.

Transformants E. coli HB101/pXar3 (coding for protein M3), E. coli HB101/pXar4 (coding for protein A4), E. coli HB101/pXar5 (coding for protein A5), E. coli HB101/pXar9 (coding for protein B9) and E. coli HB101/pXar14 (coding for protein C4) each obtained in Example 1 described below were deposited with the Institute for Fermentation, Osaka, Japan (IFO) under the accession numbers IFO 14928, IFO 14929, IFO 14930, IFO 14931 and IFO 14932, respectively, on August 28, 1989. These transformants were also deposited with the Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Japan (FRI) under the Budapest Treaty under the accession numbers FERM BP-2578, FERM BP-2579, FERM BP-2580, FERM BP-2581 and FERM BP-2582, respectively, on September 2, 1989.

The transformants E. coli HB101/pXbr22 (coding for Xenopus laevis BMP-2A), E. coli HB101/pXbr23

(coding for Xenopus laevis BMP-2B) and E. coli HB101/pXbr41 (coding for protein Xenopus laevis Vgr-1) each obtained in Example 2 described below were deposited with the Institute for Fermentation, Osaka, Japan (IFO) under the accession numbers IFO 15080, IFO 15081 and IFO 15082, respectively, on August 10, 1990. These transformants were also deposited with the Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Japan (FRI) under the Budapest Treaty under the accession numbers FERM BP-3066, FERM BP-3065 and FERM BP-3067, respectively, on August 16, 1990.

Example 1

Preparation of Xenopus laevis Liver-Derived DNA Library

(1) Preparation of Xenopus laevis Chromosome DNA

The liver (1 g) of Xenopus laevis was powdered in liquid nitrogen, and 10 ml of buffer (1) [100 μg/ml proteinase K, 0.5% Sarkosil, 0.5 M EDTA (pH 8.0)] was added thereto, followed by incubation at 50° C for 2 hours. The resulting DNA sample was treated with phenol, and then dialyzed against buffer (2) [10 mM EDTA, 10 mM NaCl, 50 mM Tris-HCl (pH 8.0)] to remove phenol. RNase was added thereto to a final concentration of 100 μg/ml, and the mixture was incubated at 37° C for 3 hours, followed by phenol treatment twice. The aqueous layer was dialyzed against buffer (3) [1 mM EDTA, 10 mM Tris-HCl (pH 8.0)]. Thus, about 1 mg of liver-derived chromosome DNA was obtained. This DNA (10 μg) was partially cleaved with restriction enzyme Sau3AI, and the resulting product was subjected to equilibrium density gradient centrifugation using CsCl. Fractions containing DNA fragments having lengths of 10 to 20 kb were selected and introduced into fragments obtained by cleaving phage charon 28 DNA with BamHI and used as a vector. This reaction called "ligation" was conducted at 15° C for 16 hours. The charon 28 vector into which the Xenopus laevis chromosome DNA was thus introduced was contained in a phage head (in vitro packaging). This procedure was carried out by using a commercial packaging kit (Gigapack Gold, Stratagene). This recombinant phage was amplified by infection with E. coli LE392. Specifically, the phage was mixed with excess LE392 to allow LE392 to adsorb the phage at 37° C for 10 minutes. Then, the mixture was plated on NZYM medium (containing 13% agar), followed by incubation overnight.

(2) Screening

The total number of the phage clones was estimated to be about 1,000,000 from the number of the plaques produced in a dish. As a probe (DNA used for detection of a desired gene by hybridization), there was used rat activin βA cDNA [ Molecular Endocrinology 1 , 388-396 (1987)] labeled with $^{32}P$ by a random priming method. The plaques transcribed from the dish to a nitrocellulose membrane were returned to neutrality (0.2 M Tris, 0.6 M NaCl, pH 7.4) through alkali treatment (immersion in 0.1 N NaOH, 0.6 M NaCl for 30 seconds). After completion of the treatment described above, the membrane was heated in a vacuum thermostat at 80° C for 1 hour. After heating, the membrane was immersed in a hybridization solution (50% formamide, 5 X Denhardt's solution, 5 X SSPE, 0.1% SDS, 100 μg/ml denatured salmon sperum DNA) to incubate it at 42° C for 4 hours. Then, the membrane was allowed to stand in the mixture solution of the above hybridization solution and the DNA probe at 60° C overnight. This procedure was carried out in a plastic bag. The next day, the nitrocellulose membrane was taken out of the bag, and washed with a solution of 2 X SSC and 0.1% SDS for 15 minutes and with a solution of 0.1 X SSC and 0.1% SDS for 15 minutes, increasing the temperature stepwise, until the cpm value of the membrane reached about 1,000 cpm. After washing, the washing solution was removed by filter paper, and then the membrane was subjected to autoradiography. The plaque containing the desired gene was identified by exposure of a Fuji X-ray film. The genes were cloned by repetition of the above plaque hybridization.

20 X SSC contains 0.3 M sodium citrate (pH 7.0) and 3 M NaCl; 20 X SSPE contains 0.2 M sodium phosphate, 20 m EDTA and 3 M NaCl (pH 7.4); and Denhardt's solution contains 1% Ficoll, 1% polyvinylpyrrolidone and 1% BSA (Pentex Fraction V).

(3) Determination of Nucleotide Sequence (Sequencing)

All of the five isolated clones A4, A5, B9, C4 and M3 were each subcloned into plasmid pUC19. In subcloning each clone into plasmid pUC19, subcloning was carried out utilizing a restriction enzyme recognition site which produced a fragment hybridized with the probe for each clone. However, for cloning clone A4, a commercial Bg1II linker was used to ligate a SmaI site.

The plasmids were each transformed into competent cell HB101 ( E. coli ) prepared by the rubidium chloride method to obtain five kinds of transformants E. coli HB101/pXar3 (coding for protein M3), E. coli HB101/pxar4 (coding for protein A4), E. coli HB101/pxar5 (coding for protein A5), E. coli HB101/pXar9 (coding for protein B9) and E. coli HB101/pXar14 (coding for protein C4), respectively.

For determination of the nucleotide sequence, a deletion mutant of each clone was prepared, and the shortest of fragment hybridized with the probe was selected. The nucleotide sequence was determined from pUC19 by the direct Sanger method (or the dideoxy method).

For translation of the nucleotide sequence to an amino acid sequence or for screening of homology, a software for genetic analysis (GENETYX, Nippon SDC) was used.

| Homology at Nucleic Acid Level | | | | | | |
|---|---|---|---|---|---|---|
| TYX nucleotide | Rat Act βA, % | Rat Act βA, % | Human TGF β2, % | xVgI % | M3 % | A4 % |
| A5 | 70.3 (101) | 47.5 (314) | 43.8 (169) | 48.5 (171) | 54.7 (258) | 63.7 (328) |
| A4 | 69.5 (0.5) | - | - | - | 55.4 (251) | |
| M3 | 63.6 (332) | 53.9 (672) | 33.1 (689) | - | | |

In the above table, numerical values in parentheses indicate the length compared (bp).

| Homology at Amino Acid Level | | | | | | |
|---|---|---|---|---|---|---|
| TYX nucleotide | Rat Act βA, % | Rat Act βA, % | Human TGF β2, % | xVgI % | M3 % | A4 % |
| A5 | 58.8 (34) | 44.1 (34) | 37.2 (43) | 50.0 (38) | 26.0 (77) | 67.6 (68) |
| A4 | 41.3 (63) | 44.1 (34) | 39.5 (43) | 52.6 (38) | 30.3 (66) | |
| M3 | 50.3 (149) | 49.4 (162) | 32.8 (128) | 40.6 (106) | | |

In the above table, numerical values in parentheses indicate the length compared (bp).

Example 2

Preparation of Xenopus laevis Unfertilized Egg-Derived DNA Library

(1) Preparation of Xenopus laevis BMP-2A Probe

A probe was prepared by fragmentation of chromosome DNA Xar14 coding for Xenopus laevia BMP-2A with restriction enzymes PstI and HindIII, and three kinds of cDNAs, Xbr22, Xbr23 and Xbr41 were isolated by screening of a Xenopus laevis unfertilized egg cDNA library by a hybridization method. The comparison with the structure of the Xenopus laevis BMP chromosome DNA already isolated revealed that Xbr22, Xbr23 and Xbr41 coded for proteins having homology with Xenopus laevis BMP-2A, Xenopus laevis BMP-2B and mouse Vgr-1 reported by Lyon et al. [ Proc. Natl. Acad. Sci. U.S.A. 806 , 4554-4558 (1989)], respectively.

The Xenopus laevis unfertilized egg cDNA library was provided by the Salk Institute (C. Kintner). This

library was prepared based on λgt10. This recombinant phage was amplified by infection with E. coli NM514. Specifically, the phage was mixed with excess NM514 to allow NM514 to adsorb the phage at 37° C for 10 minutes. Then, the mixture was plated on NZYM medium (containing 13% agar), followed by incubation overnight.

(2) Screening

The total number of the phage clones was estimated to be about 1,200,000 from the number of the plaques produced in a dish. As a probe (DNA used for detection of a desired gene by hybridization), there was used a DNA fragment (185 bp) obtained by cleaving Xar14 with restriction enzymes PstI and HindIII and labeled with $^{32}P$ by a random priming method. The plaques transcribed from the dish to a nitrocellulose membrane were returned to neutrality (0.2 M Tris, 0.6 M NaCl, pH 7.4) through alkali treatment (immersion in 0.1 N NaOH, 0.6 M NaCl for 30 seconds). After completion of the treatment described above, the membrane was heated in a vacuum thermostat at 80° C for 1 hour. After heating, the membrane was immersed in a hybridization solution (50% formamide, 5 X Denhardt's solution, 5 X SSPE, 0.1% SDS, 100 µg/ml denatured salmon sperm DNA) to incubate it at 42° C for 4 hours. Then, the membrane was allowed to stand in the mixture solution of the above hybridization solution and the DNA probe at 60° C overnight. This procedure was carried out in a plastic bag. The next day, the nitrocellulose membrane was taken out of the bag, and washed with a solution of 2 X SSC and 0.1% SDS for 15 minutes, increasing the temperature stepwise, until the cpm value of the membrane reached about 1,000 cpm. After washing, the washing solution was removed by filter paper, and then the membrane was subjected to autoradiography. The plaque containing the desired gene was identified by exposure of a Fuji X-ray film. The genes were cloned by repetition of the above plaque hybridization.

20 X SSC contains 0.3 M sodium citrate (pH 7.0) and 3 M NaCl; 20 X SSPE contains 0.2 M sodium phosphate, 20 m EDTA and 3 M NaCl (pH 7.4); and Denhardt's solution contains 1% Ficoll, 1% polyvinylpyrrolidone and 1% BSA (Pentex Fraction V).

(3) Determination of Nucleotide Sequence (Sequencing)

All of the three isolated clones Xbr22, Xbr23 and Xbr41 were each subcloned into plasmid pUC19. In subcloning each clone into plasmid pUC19, subcloning was carried out utilizing a restriction enzyme recognition site which produced a fragment hybridized with the probe for each clone.

The plasmids were each transformed into competent cell HB101 ( E. coli ) prepared by the rubidium chloride method to obtain three kinds of transformants E. coli HB101/pXbr22 (coding for Xenopus laevis BMP-2A), E. coli HB101/pXbr23 (coding for Xenopus laevis BMP-2B) and E. coli HB101/pXbr41 (coding for protein Xenopus laevis Vgr-1), respectively.

For determination of the nucleotide sequence, a deletion mutant of each clone was prepared, and the shortest fragment that hybridized with the probe was selected. The nucleotide sequence was determined from pUC19 by the direct Sanger method (or the dideoxy method).

For translation of the nucleotide sequence to an amino acid sequence or for screening of homology, a software for genetic analysis (GENETYX, Nippon SDC) was used.

Figs. 2(6) to 2(8) show the respective nucleotide sequences, and Figs. 4(VI) to 4(VIII) show the respective amino acid sequences.

Example 3

In order to examine the biological activity of the Xenopus laevis BMP-related gene products, each of Xbr22, Xbr23 and Xbr41 cDNAs was inserted into expression vector pCDM8 (Invitrogen, U.S.A.) for animal cells, and expressed in a COS cell(African green monkey kidney cell). The resulting culture supernatant was used for determination of the biological activity.

Each of the Xbr22, Xbr23 and Xbr41 cDNAs to which XhoI linkers were ligated at both ends thereof was inserted into the XhoI restriction enzyme-cleaving site of pCDM8 to use it for transfection (introduction of DNA). 3 X $10^6$ cells were subcultured in a 100 mm diameter plastic dish, and the medium was removed after 24 hours, followed by washing once with 10 ml of TBS (Tris-buffered saline). 300 µl of a DNA solution (1.5 µg DNA) diluted with TBS was mixed with 300 µl of a 0.1% DEAE-dextran solution, and the combined

solution was added dropwise to the cells. After standing at ordinary temperature for 15 minutes, the cells were washed once with 300 µl of TBS, and then incubated in Dulbecco's modified Eagle's medium (DMEM, containing 10% FBS, 100 U/ml penicillin, 100 mcg/ml streptomycin and 100 uM chloroquine). After 3 hours, the cells were washed twice with TBS and incubated in DMEM (containing 10% FBS, 100 U/ml penicillin and 100 mcg/ml streptomycin). After 24 hours, the cells were washed three times with TBS and incubated in DMEM (containing 100 U/ml penicillin and 100 mcg/ml streptomycin) for 4 days, followed by recovery of the medium. The recovered medium was centrifuged at 2,000 rpm for 5 minutes to obtain a culture supernatant.

The culture supernatant thus obtained was used for determination of the biological activity as a sample containing Xenopus laevis BMP2-A, BMP-2B or protein Vgr-1. Namely, each of the samples was added to the medium of rabbit chondrocytes in monolayer cultures [Y. Kato et al., Exp. Cell Res. 130 , 73-81 (1980); Y. Kato et al., J. Biol. Chem. 265 , 5903-5909 (1990)] to examine their effect on the synthesis of proteoglycan, the main component of a cartilage matrix. As a result, the control in which the COS cell was transfected with the expression vector alone and the medium conditioned by untreated COS cells did not affect the synthesis of proteoglycan, as shown in the following table. In contrast, the above three kinds of proteins obtained in the present invention strongly promoted the synthesis of proteoglycan by the cartilage cells. The maximum activity of Xenopus laevis BMP-2A, BMP- 2B and Vgr-1 was stronger than that of TGF-beta-1. The synthesis of proteoglycan was determined by measuring $^{35}$S-sulfate incorporation into glycosaminoglycans [Y. Kato et al., Exp. Cell Res. 130 , 73-81 (1980); Y. Kato et al., J. Biol. Chem. 265 , 5903-5909 (1990)]. These results show that the BMPs of Xenopus laevis promote the differentiation of cartilages, and suggest that the BMPs of other animals have similar effects. The BMPs are therefore expected to be applied to therapeutic agents for healing acceleration of fractures and for various diseases of cartilages and bones (such as arthritis and osteoporosis).

* Kind of Cell

Rabbit costal chondrocytes maintained on 6-mm diameter plastic wells.

* Kind of Marker

S lµCi/ in 100 µl medium per well

* Kind of Medium

A 1:1 (V/V) mixture of DMEM and Ham's F-12 medium supplemented with 0.3% fetal bovine serum.

| No. | Additive | Count | | | Mean ± S.D. | % to Control |
|---|---|---|---|---|---|---|
| 1 | Control | 5193 | 4328 | 4269 | 4695 ± 351 | 100 |
| | | 4565 | 4727 | 5089 | | |
| 2 | xBMP2A 1 5µl | 2362 | 2749 | 2758 | 2362 ± 185 | 56 |
| 3 | xBMP2A 1/3 5µl | 12198 | 15502 | 21891 | 16530 ± 4023 | 352 |
| 4 | xBMP2A 1/10 5µl | 10004 | 9738 | 8848 | 9530 ± 494 | 203 |
| 5 | xBMP2B 1 5µl | 3171 | 2906 | 3219 | 3099 ± 138 | 66 |
| 6 | xBMP2B 1/3 5µl | 11315 | 9750 | 13139 | 11401 ± 1385 | 243 |
| 7 | xBMP2B 1/10 5µl | 12426 | 13457 | 13324 | 13069 ± 458 | 278 |
| 8 | xVgr-1 1 5µl | 5188 | 2833 | 4416 | 4146 ± 980 | 88 |
| 9 | xVgr-1 1/3 5µl | 7486 | 8834 | 7202 | 7841 ± 712 | 167 |
| 10 | xVgr-1 10 5µl | 15286 | 15645 | 13032 | 14654 ± 1156 | 312 |
| 11 | pCDM8 5µl | 3604 | 2694 | 2927 | 3075 ± 386 | 65 |
| 12 | pCMD8 1µl | 2637 | 4219 | | 3428 ± 791 | 73 |
| 13 | DNA(-) 5µl | 3625 | 4050 | 4714 | 4130 ± 448 | 88 |
| 14 | DNA(-) 1µl | 5695 | 4657 | | 5176 ± 519 | 110 |
| 15 | DME 5µl | 3614 | 8963 | 3850 | 5476 ± 2468 | 117 |
| 16 | DME 1µl | 4384 | 3874 | 5760 | 4675 ± 799 | 100 |
| 17 | TGF-B1 3ng/ml | 9381 | 12474 | 10922 | | |
| | | 10058 | 11546 | 11155 | 10923 ± 998 | 233 |
| 18 | Ins. 5 g/ml | 19431 | 20476 | 22746 | | |
| | | 25066 | 27835 | 24965 | 23420 ± 2876 | 499 |
| 19 | Ins. 3 g/ml | 13620 | 15378 | 11987 | | |
| | | 11240 | 12699 | 12666 | 12932 ± 1313 | 275 |

pCDM8: A culture solution of the cells into which pCDM8 is introduced as a vector

DNA(-): A culture solution which is in contact with the cells, which do not produce the BMPs

DME: A solution which is not in contact with the cells

Ins.: Insulin

Experiments Procedure

Rabbit chondrocytes were isolated from growth plates of ribs of 3- to 4- week old male New Zealand rabbits, as previously described (Y. Kato et al. Exp. Cell Res. ). Cells were seeded at a density $10^4$ cells / 6-mm diameter plastic culture well in 0.1 ml of Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum and antibiotics. When cultures became confluent, the cells were preincubated for 24 hours in 0.1 ml of a 1:1 mixture of DMEM and Ham's F-12 medium supplemented with 0.3% fetal bovine serum (DF). The cells were then transferred to 0.1 ml of the same medium (DF) supplemented with 1 or 5 µl of the medium that was conditioned by various COS cells: [The conditioned medium was diluted or not diluted with DMEM (a final concentration of 10 or 30%)]. After 3 hours, 5 µl of DMEM supplemented with lµCi of $^{35}SO_4^{2-}$ was also added, and incubation was continued for a further 17 hours (Y. Kato et al. Exp. Cell Res. ).

## Claims

1. A Xenopus laevis bone morphogenetic protein.

2. A Xenopus laevis bone morphogenetic protein in accordance with claim 1, wherein said protein is a mature protein containing an amino acid sequence corresponding to amino acid sequence represented by Nos. 15 to 130 of formula (I) shown in Fig. 3, an amino acid sequence represented by Nos. 14 to 127 of formula (II) shown in Fig. 3, an amino acid sequence represented by Nos. 6 to 119 or Nos. 22 to 119 of formula (III) shown in Fig. 3, an amino acid sequence represented by Nos. 6 to 63 of formula (IV) shown in

Fig. 3, an amino acid sequence represented by Nos. 6 to 65 of formula (V) shown in Fig. 3, an amino acid sequence represented by Nos. 282 to 398 or Nos. 298 to 398 of formula (VI) shown in Fig. 4, an amino acid sequence represented by Nos. 288 to 401 or Nos. 304 to 401 of formula (VII) shown in Fig. 4, or an amino acid sequence represented by Nos. 328 to 426 of formula (VIII) shown in Fig. 4.

3. A Xenopus laevis bone morphogenetic protein in accordance with claim 1, wherein said protein is a precursor protein containing an amino acid sequence corresponding to amino acid sequence represented by formula (I), (II), (III), (IV) or (V) shown in Fig. 3, or formula (VI), (VII) or (VIII) shown in Fig. 4.

4. A DNA comprising a DNA segment coding for a Xenopus laevis bone morphogenetic protein.

5. A DNA in accordance with claim 4, wherein said DNA segment comprises a nucleotide sequence corresponding to the nucleotide sequence represented by formula (1), (2), (3), (4), (5), (6), (7) or (8) shown in Fig. 2, or a portion thereof.

6. A transformant bearing a DNA comprising a DNA segment coding for a Xenopus laevis bone morphogenetic protein.

7. A transformant in accordance with claim 6, which has the characteristics of Escherichia coli HB101/pXar3 (FERM BP-2578).

8. A transformant in accordance with claim 6, which has the characteristics of Escherichia coli HB101/pXar4 (FERM BP-2579).

9. A transformant in accordance with claim 6, which has the characteristics of Escherichia coli HB101/pXar5 (FERM BP-2580).

10. A transformant in accordance with claim 6, which has the characteristics of Escherichia coli HB101/pXar9 (FERM BP-2581).

11. A transformant in accordance with claim 6, which has the characteristics of Escherichia coli HB101/pXar14 (FERM BP-2582).

12. A transformant in accordance with claim 6, which has the characteristics of Escherichia coli HB101/pXbr22 (FERM BP-3066).

13. A transformant in accordance with claim 6, which has the characteristics of Escherichia coli HB101/pXbr23 (FERM BP-3065).

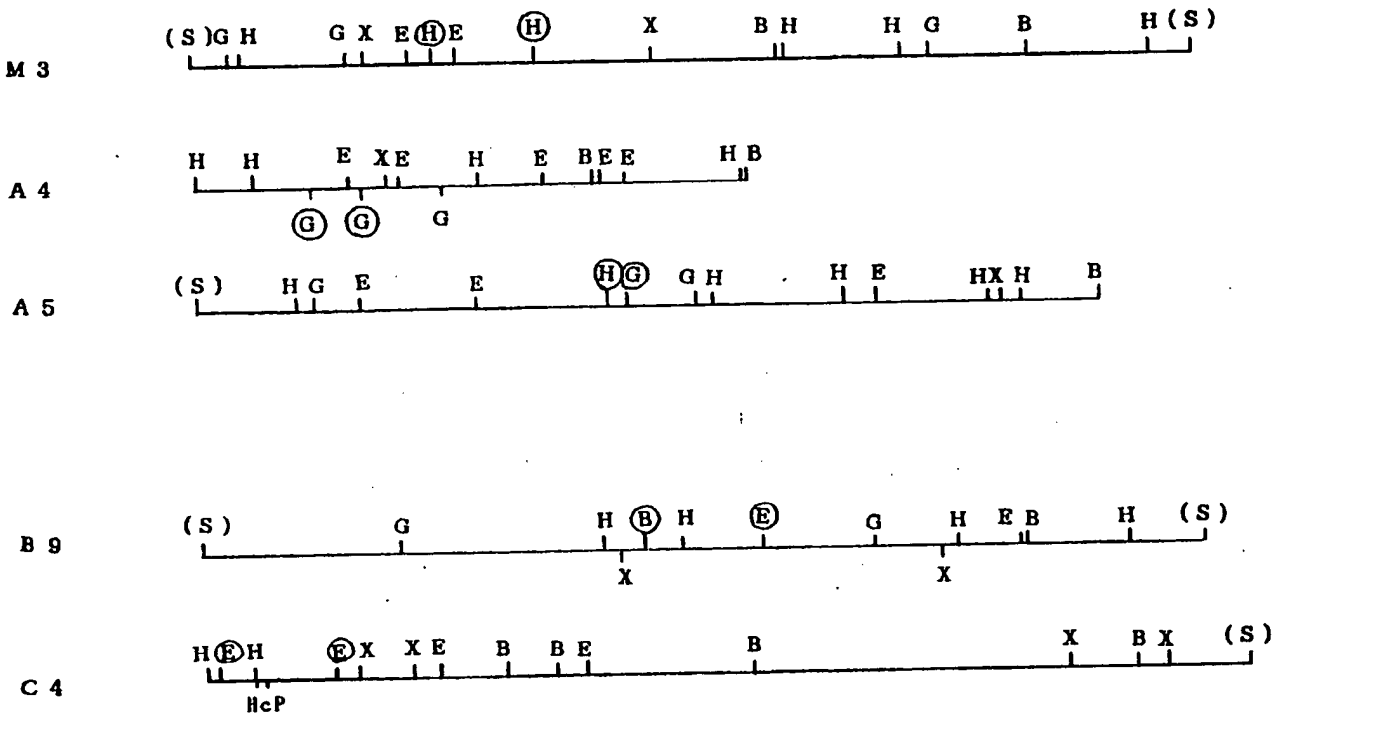
14. A transformant in accordance with claim 6, which has the characteristics of Escherichia coli HB101/pXbr41 (FERM BP-3067).

15. A method for preparing a Xenopus laevis bone morphogenetic protein which comprises culturing a transformant bearing a DNA comprising a DNA segment coding for the protein, producing and accumulating the protein in a culture, and collecting the protein thus obtained.

16. A composition for therapy of fracture or osteoporosis which contains an effective amount of a Xenopus laevis bone morphogenetic protein according to claim 1 and pharmaceutically acceptable additional components.

17. A method for promoting the synthesis of proteoglycan in cartilage cells by administering an effective amount of a Xenopus laevis bone morphogenetic protein according to claim 1 to a mammal in need thereof.

Fig. 1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1 k b

Fig. 2 (1)-1

B 9


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Fig. 2 (1)-2

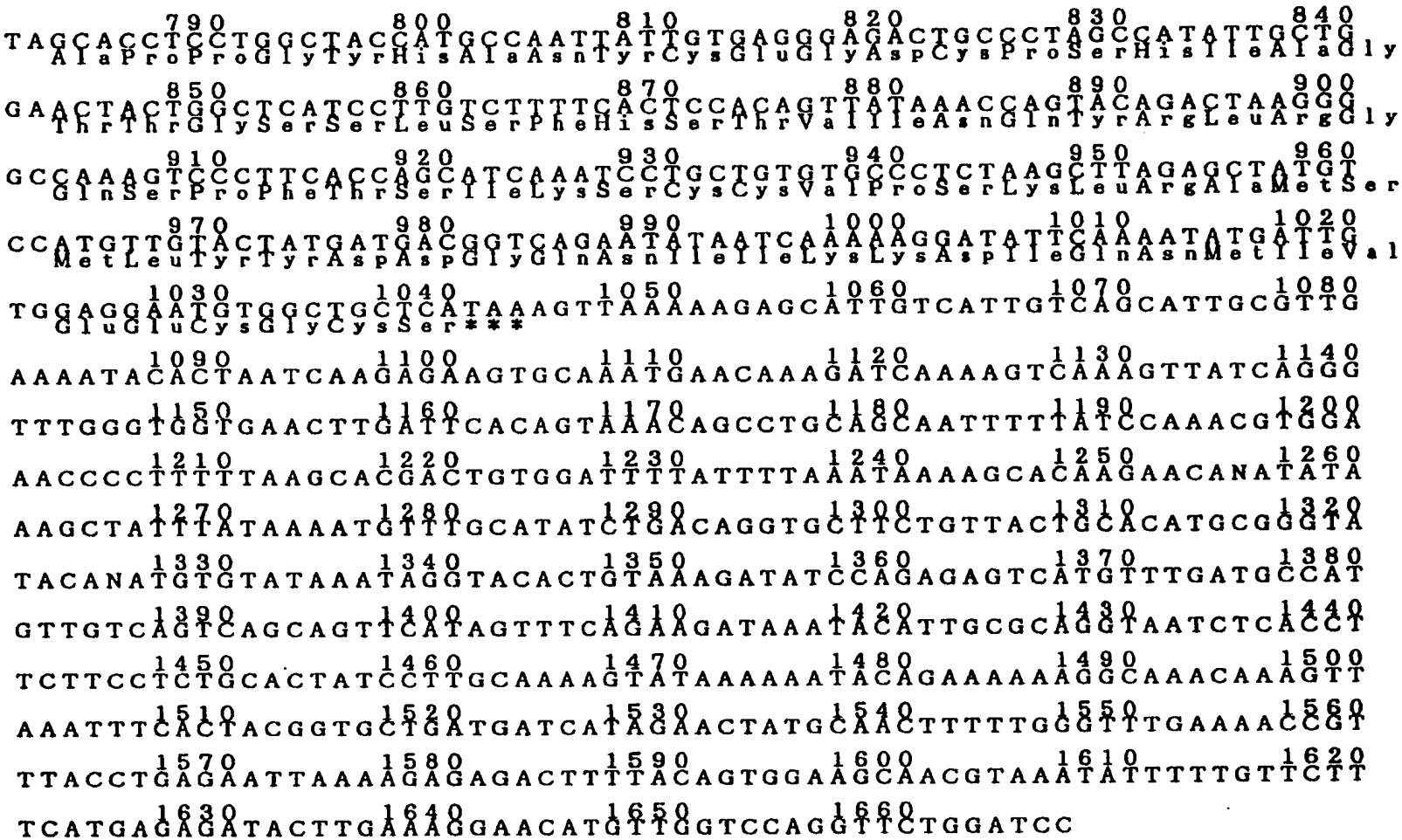
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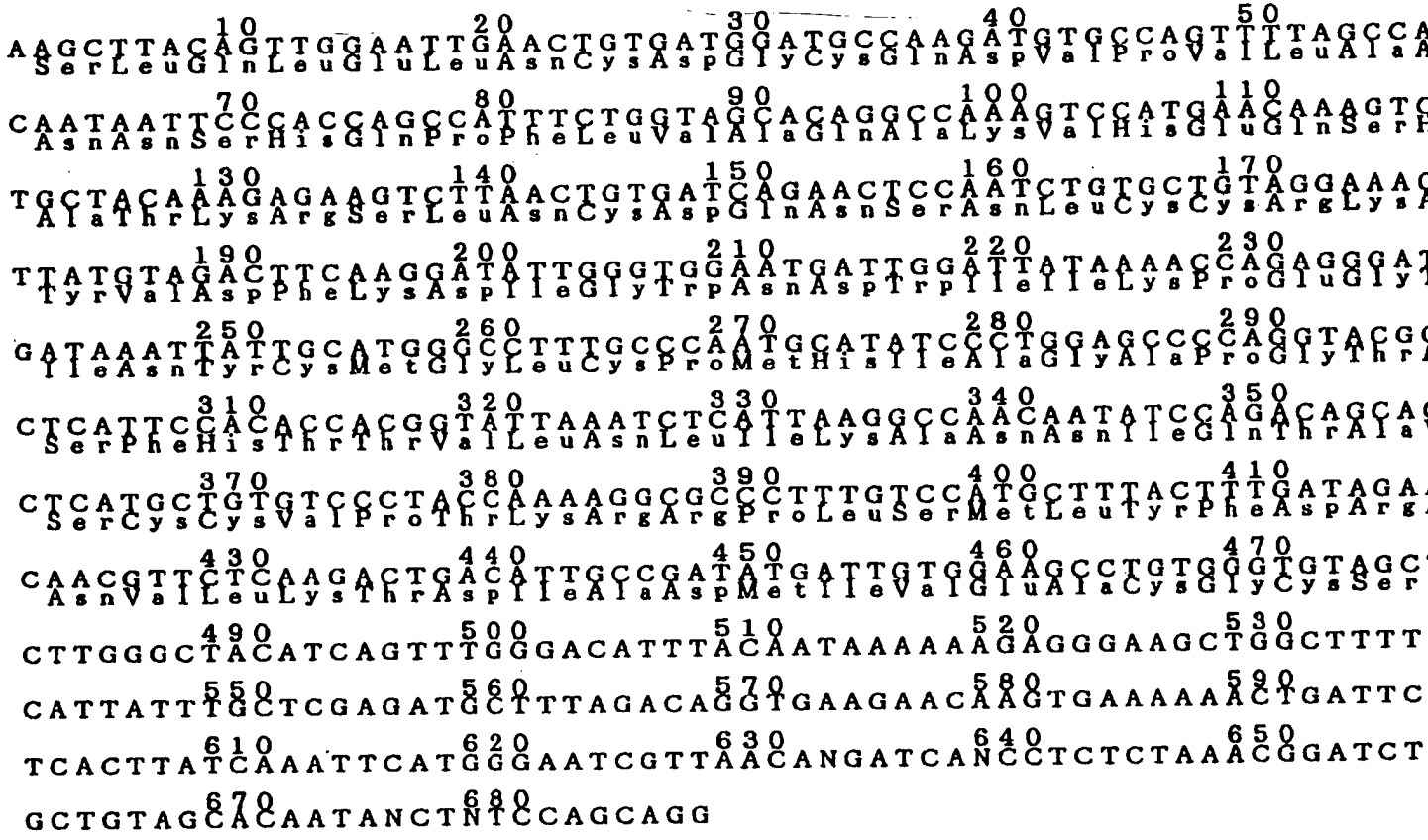
Fig. 2 (2)

M 3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AsnValLeuLysThrAspIleAlaAspMetIleValGluAlaCysGlyCysSer***
490 500 510 520 530 540
CTTGGGCTACATCAGTTTGGGACATTTACAATAAAAAAGAGGGAAGCTGGCTTTTCTCTT
550 560 570 580 590 600
CATTATTTGCTCGAGATGCTTTAGACAGGTGAAGAACAAGTGAAAAAACTGATTCCACAT
610 620 630 640 650 660
TCACTTATCAAATTCATGGGAATCGTTAACANGATCANCCTCTCTAAACGGATCTAGGGA
670 680
GCTGTAGCACAATANCTNTCCAGCAGG

Fig. 2 (3)-1

C4

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          10                  20                  30                  40                  50                  60
AGAGCAGGTCAAGAGCCCTTTGANAGTGACAGCAGCAAATTGCATCGGATTANTATTTAC

          70                  80                  90                 100                 110                 120
GACATTGTCAGGSCAGCGGNNNNCTGGSCTSCCGGGGGCCTGTTGTGAGACTATTGGACA
                                                   ArgGlyProValValArgLeuLeuAspThr

         130                 140                 150                 160                 170                 180
CCAAACTGGTACATCATAATGAAAGCAAATGGGAAAGTTTTGATGTAGCGCCGGCAATTG
   LysLeuValHisHisAsnGluSerLysTrpGluSerPheAspValAlaProAlaIleAla

         190                 200                 210                 220                 230                 240
CGCGGTGGATTGCACATAAACAGCCTAACCATGGGTTTGTTGTTGAAGTTACTCACTTGG
   ArgTrpIleAlaHisLysGlnProAsnHisGlyPheValValGluValThrHisLeuAsp

         250                 260                 270                 280                 290                 300
ACAATGACAAAAATGTGCCTAAGAAGCATGTGAGGATTAGTAGGTCTTTAACCCCGGATA
   AsnAspLysAsnValProLysLysHisValArgIleSerArgSerLeuThrProAspLys

         310                 320                 330                 340                 350                 360
AAGATAACTGGCCTCAGATACGGCCATTGTTGGTAACTTTTAGCCATGATGGTAAAGGAC
   AspAsnTrpProGlnIleArgProLeuLeuValThrPheSerHisAspGlyLysGlyHis

         370                 380                 390                 400                 410                 420
ATGCTCTTCACAAAAGACAAAAGCGCCAAGCTAGGCACAAACAACGTAAACGCCTTAAAT
   AlaLeuHisLysArgGlnLysArgGlnAlaArgHisLysGlnArgLysArgLeuLysSer

         430                 440                 450                 460                 470                 480
CGAGCTGCAGGAGGCATCCGTTGTACGTAGATTTCAGCGACGTTGGTTGGAATGACTGGA
   SerCysArgArgHisProLeuTyrValAspPheSerAspValGlyTrpAsnAspTrpIle

         490                 500                 510                 520                 530                 540
TTGTTGCCCCACCTGGGTATCATGCCTTTTACTGCCACGGGGAATGTCCTTTTCCACTGG
   ValAlaProProGlyTyrHisAlaPheTyrCysHisGlyGluCysProPheProLeuAla
```

Fig. 2(3)-2

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          550         560         570         580         590         600
CAGACCATTTAAACTCTACAAACCATGCAATCGTACAAACTTTGGTGAACTCTGTCAACA
  AspHisLeuAsnSerThrAsnHisAlaIleValGlnThrLeuValAsnSerValAsnThr

          610         620         630         640         650         660
CAAACATCCCCAAAGCTTGCTGCGTCCCCACAGAACTCAGTGCCATATCCATGCTCTATC
  AsnIleProLysAlaCysCysValProThrGluLeuSerAlaIleSerMetLeuTyrLeu

          670         680         690         700         710         720
TTGATGAGAATGAAAAAGTAGTATTAAAAAATTATCAAGACATGGTCGTGGAGGGGTGCG
  AspGluAsnGluLysValValLeuLysAsnTyrGlnAspMetValValGluGlyCysGly

          730         740         750         760         770         780
GATGCCGTTAGGCAGTTACGCGCAAGCCAGAGACAAGAAAGATGACACTTTAATATTTCC
  CysArg***

          790         800         810         820         830         840
TTTTGGAGACTATATTTATGCTTTGAAAAATGATGAAACANTTATTTTGAAAATATATTT

          850         860         870         880         890         900
ATGTCTACACGGAGGTTGGGAAGCAAATATTTTAATCAGAGAAATATTCCTTTTTTTAGT

          910         920         930         940         950
TGTACATTTTTATAAGGGTTTGTACCCAGCACATGAAGTATAATGGTCAGATTGA
```

Fig. 2 (4)

A4

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        10          20          30          40          50          60
CCTGAGANTTAAGAAGTGTGGGATTTAACAGAACAGGACGACCGACCAATGAGAAAGCTA

        70          80          90          100         110         120
TTTTTCTTGTCTNTGGTAGGACAAAGAAACGGGACNTGTTCTTCAATGAGATTAAAGCCA
                    PhePheAsnGluIleLysAlaArg

       130         140         150         160         170         180
GGTCTGGCCAAGATGACAAGACTGTCTATGAATATTTATTCAATCAGAGGAGAAAGAGAC
  SerGlyGlnAspAspLysThrValTyrGluTyrLeuPheAsnGlnArgArgLysArgArg

       190         200         210         220         230         240
GAGCTCCTCTGTCAACTAGGCAAGGGAAGAGGCCTAATAAGAATTCAAAAGCAAGATGTA
  AlaProLeuSerThrArgGlnGlyLysArgProAsnLysAsnSerLysAlaArgCysSer

       250         260         270         280         290         300
GCAAGAAACCACTTCATGTCAATTTCAAGGATATGGGTTGGGATGATTGGATTATTGCCC
  LysLysProLeuHisValAsnPheLysAspMetGlyTrpAspAspTrpIleIleAlaPro

       310         320         330         340         350
CTTTGGAGTATGAGGCATATCATTGTGAAGGGCTTTGTGAGTTCCCTCTGAGATCT
  LeuGluTyrGluAlaTyrHisCysGluGlyLeuCysGluPheProLeuArgSer
```

Fig. 2 (5)

A5

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          10          20          30          40          50          60
AAGCTTTACTGGTGGTGTCTTCCCATTCCAAGAGGAGGGAAAACTTGTTTAAGGAGATCA

          70          80          90         100         110         120
GGGACAAGATTAAGTCAATTGGAAATCCTAAATTCTTGGAGCCACCGGATTCAGTCAACA
                    IleLeuGlyAlaThrGlyPheSerGlnGln

         130         140         150         160         170         180
GTCCATCGCCAAGAGGAGATGGAAACGAACAACTCTCCCCACTAGGACAAATAATGGCAA
 SerIleAlaLysArgArgTrpLysArgThrThrLeuProThrArgThrAsnAsnGlyLys

         190         200         210         220         230         240
AGGTCATGCGAAGAAATCCAAAACAAGGTGTAGCAAGAAGCCCCTTCTTGTCAACTTCAA
 GlyHisAlaLysLysSerLysThrArgCysSerLysLysProLeuLeuValAsnPheLys

         250         260         270         280         290         300
GGAGTTGGGTTGGGATGACTGGATTATTGCTCCCTTGGATTATGAAGCCTATCACTGCGA
 GluLeuGlyTrpAspAspTrpIleIleAlaProLeuAspTyrGluAlaTyrHisCysGlu

         310         320
GGGGGTCTGTGATTTCCCACTGAGATCT
 GlyValCysAspPheProLeuArgSer
```

Fig. 2 (6)
pxbr22 (BMP2A)

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      10        20        30        40        50        60        70        80        90       100
GAATTCTCTTCCCTCTCACCGGCCTCTCGTCTCTACTCACCTCCCGGCGACCCCGGCTGGACTGAGACACTCGCTGCCACTATGTGCGACAACTCACCGA

     110       120       130       140       150       160       170       180       190       200
CTGGGCTCGACTGGACGCGCGGACTTGTCTCCCTCCTCTGGGGACCAGCGACTTGAACTAAAGACTCGAGTGATTGTGGAAAAAACACGCGGGGAGCAGA

     210       220       230       240       250       260       270       280       290       300
AAACCCACATCGAGACACAAACTCGGCGACTAAATCGCTCAGGTTGACAATGGTCGCTGGGATCCACTCTCTGCTCCTGCTGCAGTTTTACCAGATCTTG
                                                  M  V  A  G  I  H  S  L  L  L  L  Q  F  Y  Q  I  L

     310       320       330       340       350       360       370       380       390       400
CTGAGCGGCTGCACCGGGCTCGTCCCAGAGGAAGGCAAACGCAAGTATTCCGAATCCACTCGCTCGTCTCCGCAGCAGTCCCAACAAGTCCTCGACCAGT
L  S  G  C  T  G  L  V  P  E  E  G  K  R  K  Y  S  E  S  T  R  S  S  P  Q  Q  S  Q  Q  V  L  D  Q  F

     410       420       430       440       450       460       470       480       490       500
TTGAGCTTCGGCTGCTCAATATGTTCGGCTTGAAGAGGAGGCCGACGCCTGGCAAAAATGTTGTGATCCCCCCCCTACATGTTGGACTTGTACCACCTGCA
  E  L  R  L  L  N  M  F  G  L  K  R  R  P  T  P  G  K  N  V  V  I  P  P  Y  M  L  D  L  Y  H  L  H

     510       520       530       540       550       560       570       580       590       600
CTCGGCTCAGTTGGCCGATGATCAAGGAAGTTCTGAGGTGGACTATCACATGGAGCGGGCGGCTAGCAGAGCCAACACAGTGAGGAGCTTTCACCATGAA
S  A  Q  L  A  D  D  Q  G  S  S  E  V  D  Y  H  M  E  R  A  A  S  R  A  N  T  V  R  S  F  H  H  E

     610       620       630       640       650       660       670       680       690       700
GAATCCATGGAAGAAATTCCAGAGTCTGGTGAGAAAACAATCCAACGATTCTTCTTCAACCTTTCTTCAATTCCAGATGAGGAGCTGGTCACGTCTTCTG
E  S  M  E  E  I  P  E  S  G  E  K  T  I  Q  R  F  F  F  N  L  S  S  I  P  D  E  E  L  V  T  S  S  E

     710       720       730       740       750       760       770       780       790       800
AGCTCCGGATTTTTCGAGAGCAGGTCCAAGAGCCATTTAAGACTGACGGCAGCAAACTTCATCGGATTAATATTTATGACATTGTCAAGCCAGCGGCGGC
  L  R  I  F  R  E  Q  V  Q  E  P  F  K  T  D  G  S  K  L  H  R  I  N  I  Y  D  I  V  K  P  A  A  A

     810       820       830       840       850       860       870       880       890       900
TGCCTCCCGGGGCCCTGTTGTAAGACTATTGGACACCAGACTGATCCATCATAATGAAAGCAAATGGGAAAGTTTTGATGTGACGCCGGCAATTACACGG
A  S  R  G  P  V  V  R  L  L  D  T  R  L  I  H  H  N  E  S  K  W  E  S  F  D  V  T  P  A  I  T  R

     910       920       930       940       950       960       970       980       990      1000
TGGATTGCACATAAACAGCCTAACCATGGGTTTGTTGTTGAAGTGACTCACTTGGACAATGACACAAATGTGCCCAAGAGGCATGTGAGGATTAGTAGGT
W  I  A  H  K  Q  P  N  H  G  F  V  V  E  V  T  H  L  D  N  D  T  N  V  P  K  R  H  V  R  I  S  R  S

    1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
CTTTAACCCTGGATAAAGGTCACTGGCCTCGGATACGGCCATTATTGGTAACTTTTAGCCATGATGGCAAAGGACATGCTCTTCACAAAAGACAAAAACG
  L  T  L  D  K  G  H  W  P  R  I  R  P  L  L  V  T  F  S  H  D  G  K  G  H  A  L  H  K  R  Q  K  R

    1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
GCAAGCTAGGCACAAACAACGTAAACGCCTTAAATCGAGCTGCAGGAGGCATCCGTTGTACGTAGATTTCAGTGACGTTGGTTGGAATGACTGGATTGTT
  Q  A  R  H  K  Q  R  K  R  L  K  S  S  C  R  R  H  P  L  Y  V  D  F  S  D  V  G  W  N  D  W  I  V

    1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
GCCCCACCTGGGTATCATGCCTTTTACTGCCACGGGGAATGTCCTTTTCCACTGGCAGACCATTTAAACTCTACAAACCATGCAATCGTACAAACTTTGG
A  P  P  G  Y  H  A  F  Y  C  H  G  E  C  P  F  P  L  A  D  H  L  N  S  T  N  H  A  I  V  Q  T  L  V

    1310      1320      1330      1340      1350      1360      1370      1380      1390      1400
TGAATTCCGTCAACACAAACATTCCCAAAGCTTGCTGCGTCCCCACAGAACTCAGTGCCATCTCCATGCTCTATCTTGATGAGAATGAAAAAGTAGTATT
  N  S  V  N  T  N  I  P  K  A  C  C  V  P  T  E  L  S  A  I  S  M  L  Y  L  D  E  N  E  K  V  V  L

    1410      1420      1430      1440      1450      1460      1470      1480      1490      1500
AAAGAATTATCAAGACATGGTCGTGGAGGGGTGCGGGTGCCGTTAGGCGGGGACACACAAGCCAGAGACAAGAAAGCTGACACTTTAATATTTCCTTTTG
  K  N  Y  Q  D  M  V  V  E  G  C  G  C  R  *

    1510      1520      1530      1540      1550      1560      1570      1580      1590      1600
GAGACTATATTTATGCTTTGAAAAATGATGAAACAATTATTTTGAAAATATATTTATGTCTACACGGAGGCTGGGAAGCAAATATTTTAATCAGAGAAAT

    1610      1620      1630      1640      1650      1660      1670      1680      1690      1700
ATTCCTTTTTAGTTGTACATTTTTATAAGGGTTTGTACCCAGCACATGAAGTATAATGGTCAGATTCCTATTTTGTATTTATTTACCATTATAACCACTT

    1710      1720      1730      1740      1750      1760      1770      1780      1790      1800
TTTAAGGAAAAAAATAGCTGTTTTGTATTTATATGTAATCAACAGAGAAAATATAGGGTTTGTAAATATGTTACTGAAAGTGTTTTTTTCTTCTTTTTTT

    1810      1820      1830      1840      1850      1860      1870      1880      1890      1900
TAAATTATGTATACACAGCTGGTTATATGGCAAGTTTTTTATATTTTCTATAAAGCTAATTTCAAGGTCATTAGTTATAAACTTGATGATGTGTTGGTTC

    1910      1920      1930      1940      1950      1960      1970      1980      1990
ATTGGTAAATCCTCCATATTGTGCAATTAACATGCATTTTTATAATGTACGAAGTCCAGTCCATTGTGCATTGCTTTGCAAATTTAGAATTC
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Fig. 2 (7)
pxbr23 (BMP2B)

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      10        20        30        40        50        60        70        80        90       100
GGAATTCCGGCCCCACTGAGCTTTTCCACACATTTTTTGTGTCCAACATTGGCTGTCAAGAATCATGGAATGTTTTTCTATGCCTTGTTTTCTGTCAAGA

     110       120       130       140       150       160       170       180       190       200
CATCATGATTCCTGGTAACCGAATGCTGATGGTCATTTTATTAAGCCAAGTCCTGCTCGGAGGCACTAACTATGCCAGCCTGATACCTGACACGGGCAAG
   M  I  P  G  N  R  M  L  M  V  I  L  L  S  Q  V  L  L  G  G  T  N  Y  A  S  L  I  P  D  T  G  K

     210       220       230       240       250       260       270       280       290       300
AAGAAAGTCGCGGCCGACATTCAGGGAGGAGGTCGCAGGTCGCCTCAGAGCAATGAGCTCTTGCGGGATTTCGAGGTGACGCTGCTGCAGATGTTCGGAC
K  K  V  A  A  D  I  Q  G  G  G  R  R  S  P  Q  S  N  E  L  L  R  D  F  E  V  T  L  L  Q  M  F  G  L

     310       320       330       340       350       360       370       380       390       400
TCCGCAAGCGGCCGCAGCCCAGTAAGGATGTGGTGGTTCCCGCTTATATGCGCGACCTGTACAGGCTTCAGTCAGCGGAGGAGGAGGATGAACTGCACGA
  R  K  R  P  Q  P  S  K  D  V  V  V  P  A  Y  M  R  D  L  Y  R  L  Q  S  A  E  E  E  D  E  L  H  D

     410       420       430       440       450       460       470       480       490       500
TATCAGCATGGAGTACCCCGAGACACCCACCAGCCGCGCCAACACCGTGAGGAGCTTCCATCACGAGGAACATTTGGAGAATCTACCAGGCACAGAAGAA
 I  S  M  E  Y  P  E  T  P  T  S  R  A  N  T  V  R  S  F  H  H  E  E  H  L  E  N  L  P  G  T  E  E

     510       520       530       540       550       560       570       580       590       600
AATGGAAATTTCCGTTTTGTGTTCAACCTCAGCAGCATTCCAGAGAATGAGGTGATTTCTTCAGCAGAACTGAGACTCTATAGAGAACAAATAGACCATG
N  G  N  F  R  F  V  F  N  L  S  S  I  P  E  N  E  V  I  S  S  A  E  L  R  L  Y  R  E  Q  I  D  H  G

     610       620       630       640       650       660       670       680       690       700
GTCCAGCGTGGGATGAGGGTTTCCACCGGATAAATATATATGAAGTTATGAAACCCATCACAGCAAACGGACACATGATAAATAGGCTGCTGGACACGAG
  P  A  W  D  E  G  F  H  R  I  N  I  Y  E  V  M  K  P  I  T  A  N  G  H  M  I  N  R  L  L  D  T  R

     710       720       730       740       750       760       770       780       790       800
GGTAATCCACCACAATGTGACACAGTGGGAAAGTTTTGATGTAAGCCCTGCAATTATGAGGTGGACCCTGGATAAACAGATAAACCATGGGCTTGCCATT
 V  I  H  H  N  V  T  Q  W  E  S  F  D  V  S  P  A  I  M  R  W  T  L  D  K  Q  I  N  H  G  L  A  I

     810       820       830       840       850       860       870       880       890       900
GAGGTCATTCACCTCAACCAAACAAAAACTTATCAGGGGAAGCATGTAAGGATAAGTCGATCTTTATTACCTCAAAAGGATGCAGACTGGTCACAGATGA
E  V  I  H  L  N  Q  T  K  T  Y  Q  G  K  H  V  R  I  S  R  S  L  L  P  Q  K  D  A  D  W  S  Q  M  R

     910       920       930       940       950       960       970       980       990      1000
GACCACTTTTAATTACATTCAGCCATGATGGCAGGGGGCATGCACTGACTAGGAGGTCAAAAAGAAGTCCAAAACAGCAGAGACCCCGTAAAAAAAATAA
  P  L  L  I  T  F  S  H  D  G  R  G  H  A  L  T  R  R  S  K  R  S  P  K  Q  Q  R  P  R  K  K  N  K

    1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
ACACTGCCGGAGACATTCTCTTTATGTGGATTTCAGCGATGTGGGCTGGAATGATTGGATTGTGGCACCTCCTGGATACCAGGCCTTTTACTGCCATGGA
 H  C  R  R  H  S  L  Y  V  D  F  S  D  V  G  W  N  D  W  I  V  A  P  P  G  Y  Q  A  F  Y  C  H  G

    1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
GATTGTCCATTTCCCTTGGCTGATCACCTAAACTCAACTAACCATGCTATTGTACAAACTCTGGTAAACTCTGTTAACTCAAGCATCCCAAAAGCATGCT
D  C  P  F  P  L  A  D  H  L  N  S  T  N  H  A  I  V  Q  T  L  V  N  S  V  N  S  S  I  P  K  A  C  C

    1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
GCGTCCCCACAGAACTGAGTGCTATCTCCATGCTTTATTTGGATGAATATGACAAAGTCGTCCTTAAAAACTACCAGGAGATGGTGGTGGAAGGGTGTGG
  V  P  T  E  L  S  A  I  S  M  L  Y  L  D  E  Y  D  K  V  V  L  K  N  Y  Q  E  M  V  V  E  G  C  G

    1310      1320      1330      1340      1350      1360      1370      1380      1390      1400
GTGCCGTTGAGTCTGAGATCCAAACAAAAGACTGTTAACGGCTGGACTTCTTTCCACTGAACATTCACCTTGACCTTATTTATGACTTTTATGTGTAAAT
 C  R  *

    1410      1420      1430      1440      1450      1460      1470      1480      1490      1500
GTTTTTTTGACAATATGATCATATATTTTGACAAAATATATTTATAACTACGTATTAAAAGAAAAAAAAAAAAATAAAATAAGTCATTATTTTAAACATAA

    1510      1520      1530      1540      1550
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACGGAATTC
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Fig. 2 (8)
pxbr41 (Ver1)

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      10        20        30        40        50        60        70        80        90       100
GAATTCCGATATGGAATGTAAAAATACTGGTGAATTATGGGAAGTCCGACACAGACCACTAACTTCAGCATCTTATCTTTGACAAAATGAATGCTTTGAC
                                                                                  M  N  A  L  T

     110       120       130       140       150       160       170       180       190       200
AGTAAAGAGAAGATTGCCTGTGCTGCTTTTTCTTTTTCACATTTCACTGAGTTCCATCTCGTCAAATACAATATTGGAGAATGATTTCCACTCTAGTTTT
 V  K  R  R  L  P  V  L  L  F  L  F  H  I  S  L  S  S  I  S  S  N  T  I  L  E  N  D  F  H  S  S  F

     210       220       230       240       250       260       270       280       290       300
GTCCAGAGAAGACTAAAAGGCCACGAACGCAGAGAGATTCAAAAAGAGATCTTGACTATTTTAGGTTTGCAACACAGACCAAGGCCATATTTACCGGAGA
 V  Q  R  R  L  K  G  H  E  R  R  E  I  Q  K  E  I  L  T  I  L  G  L  Q  H  R  P  R  P  Y  L  P  E  K

     310       320       330       340       350       360       370       380       390       400
AAAAGAAGTCTGCACCATTATTCATGATGGATTTATACAATGCAGTAAATATTGAAGAGATGCATGCTGAAGATGTTTCCTACAGCAATAAGCCGATCTC
  K  K  S  A  P  L  F  M  M  D  L  Y  N  A  V  N  I  E  E  M  H  A  E  D  V  S  Y  S  N  K  P  I  S

     410       420       430       440       450       460       470       480       490       500
CCTAAATGAAGCTTTTTCACTGGCCACTGACCAAGAGAATGGCTTTCTTGCACATGCCGACACAGTTATGAGTTTTGCTAATTTAGTTGACAATGACAAC
 L  N  E  A  F  S  L  A  T  D  Q  E  N  G  F  L  A  H  A  D  T  V  M  S  F  A  N  L  V  D  N  D  N

     510       520       530       540       550       560       570       580       590       600
GAATTGCATAAAAACTCCTATCGCCAAAAATTCAAGTTTGATCTAACTGATATCCCACTTGGAGATGAACTGACAGCCGCTGAATTTCGAATTTATAAAG
 E  L  H  K  N  S  Y  R  Q  K  F  K  F  D  L  T  D  I  P  L  G  D  E  L  T  A  A  E  F  R  I  Y  K  D

     610       620       630       640       650       660       670       680       690       700
ATTATGTACAAAATAACGAGACATACCAGGTCACCATCTACCAGGTGCTTAAGAAGCAAGCCGACAAAGATCCTTATCTTTTCCAGGTAGACTCAAGAAC
  Y  V  Q  N  N  E  T  Y  Q  V  T  I  Y  Q  V  L  K  K  Q  A  D  K  D  P  Y  L  F  Q  V  D  S  R  T

     710       720       730       740       750       760       770       780       790       800
CATCTGGGGCACAGAAAAGGGATGGCTGACGTTTGATATTACTGCAACTGGTAATCACTGGGTGATGAACCCACATTACAACCTTGGATTGCAGTTATCA
 I  W  G  T  E  K  G  W  L  T  F  D  I  T  A  T  G  N  H  W  V  M  N  P  H  Y  N  L  G  L  Q  L  S

     810       820       830       840       850       860       870       880       890       900
GTAGAGAGTATGGATATGCAAAATGTTAATCCCAGGCTTGTGGGCCTTGTTGGAAAGAATGGTCCTCAAGACAAACAGCCATTTATGGTGGCATTCTTTA
 V  E  S  M  D  M  Q  N  V  N  P  R  L  V  G  L  V  G  K  N  G  P  Q  D  K  Q  P  F  M  V  A  F  F  K

     910       920       930       940       950       960       970       980       990      1000
AGACCTCAGATATCCATCTCCGCAGTGTTCGATCTACTAGCAATAAGCACTGGAATCAGGAAAGAGCCAAGACCTACAAGGAGCAAGATAATTTACCTCC
  T  S  D  I  H  L  R  S  V  R  S  T  S  N  K  H  W  N  Q  E  R  A  K  T  Y  K  E  Q  D  N  L  P  P

    1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
AGCAAATATTACTGATGGCATCATGCCCCCTGGAAAACGTCGTTTTTTAAAGCAAGCTTGCAAGAAACATGAACTGTTTGTAAGTTTCCGCGATCTTGGT
 A  N  I  T  D  G  I  M  P  P  G  K  R  R  F  L  K  Q  A  C  K  K  H  E  L  F  V  S  F  R  D  L  G

    1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
TGGCAAGACTGGATAATTGCACCTGAAGGATATGCTGCCTACTATTGTGATGGAGAATGTGCTTTCCCACTTAACTCTTTCATGAATGCCACAAACCATG
 W  Q  D  W  I  I  A  P  E  G  Y  A  A  Y  Y  C  D  G  E  C  A  F  P  L  N  S  F  M  N  A  T  N  H  A

    1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
CCATTGTACAAACGTTGGTACATTTCATTAACCCAGAGACTGTCCCTAAGCCATGCTGTGCACCAACTCAGCTCAATGGTATTTCTGTTTTATACTTTGA
  I  V  Q  T  L  V  H  F  I  N  P  E  T  V  P  K  P  C  C  A  P  T  Q  L  N  G  I  S  V  L  Y  F  D

    1310      1320      1330      1340      1350      1360      1370      1380      1390      1400
TGACAGTGCCAATGTTATATTAAAGAAATACAAAAATATGGTGGTTCAAGCCTGTGGTTGCCATTGACAATAGCAGTTATTCTGTTTTTAACAGTCATTT
 D  S  A  N  V  I  L  K  K  Y  K  N  M  V  V  Q  A  C  G  C  H  *

    1410      1420      1430      1440      1450      1460      1470      1480      1490      1500
TAATGGTATTGTCCTTATCGTTTATTTTAAAGTAGAGATACTTGACCATCACACTTAAAAAAATGCATTGTACACCTTAACGGATGAAAAGATTTTGTTT

    1510
TTGCATGATTTCGGAATTC
```

Fig. 3

```
βA        ARQSEDHPHRRRRR--GLECDGKVNICCKKQFFVSFKDIGWNDWIIAPSGYHANYCEGECPSHIAG
B9(I)     ...TDE.....KK.--.........S.....H.Y.........S.......P.........D.......
M3(II)    AK-VHEQS.HATK.--S.N..QNS.L..R.DYY.D.............K.E..QI...M.L..M....
βB        ..LGDSR-..I.K.--.......RTSL..RQ...ID.RL..........T..YG.....S..AYL..
BMP2A     K.EKRQAK.KQ.K.---.----.SS-.KRHPLY.D.S.V......V..P....F..H....FPL.D
C4(III)   K..KRQAR.KQ.K.---.----.SS-.RRHPLY.D.S.V......V..P....F..H....FPL.D
DPPC      ...-HARRPT..KN---H----DDT-.RRHSLY.D.S.V..DD..V..L..D.Y..H.K..FPL.D
Vg1       TLNPLRCKRP..K.SYSKLPFTAS...K.RHLY.E...V..QN.V...Q..M....Y....YPLTE
A4(IV)    R.KRRAPLST.--QGKRPNKNS.AR-.S..PLH.N...M..D......LE.E.YH...L.EFPLRS
A5(V)     R.WKRTTLPT.TNNGK.HAKKS.TR-.S..PLL.N..EL..D......LD.E.YH...V.DFPLRS
CONSENSUSE.........................C.........F...GW..W...P..Y....C.G.C......

βA        TSGSSLSFHSTVINHYRMRGHSPFANLKSCCVPTKLRPMSMLYYDDGQNIIKKDIQNMIVEECGCS
B9(I)     .T.............Q..L..Q...TSI......S...A.............................
M3(II)    AP.TAA...T..L.LIK--ANNIQTAVN.......R..L....F.RNN.VL.T..AD....A....
βB        VP..AS...TA.V.Q.....LN.-GPVN...I....SS.....F..EY..V.R.VP.........A
BMP2A     H--LNSTN.AI.QTLVN--SVNSK-IP.A.....E.SAI....L.ENEKVVL.NY.D.V..G...R
C4(III)   H--LNSTN.AI.QTLVN--SVNTN-IP.A.....E.SAI....L.ENEKVVL.NY.D.V..G...R
DPPC      H--FNSTN.AV.QTLVN-NMNPGK-VP.A.....QLDSVA...LN.QSTVVL.NY.E.T.VG...R
Vg1       I--LNG.N.AILQTLVH--SIE.EDIPLP......MS.I...F..NND.VVLRHYE..A.D....R
               ***
CONSENSUS .........H.....................CC.P.......ML..D.............M.V..CGC.
```

Fig. 4-1

(VI) BMP2A

M V A G I H S L L L L Q F Y Q I L

L S G C T G L V P E E G K R K Y S E S T R S S P Q Q S Q Q V L D Q F

E L R L L N M F G L K R R P T P G K N V V I P P Y M L D L Y H L H

S A Q L A D D Q G S S E V D Y H M E R A A S R A N T V R S F H H E

E S M E E I P E S G E K T I Q R F F F N L S S I P D E E L V T S S E

L R I F R E Q V Q E P F K T D G S K L H R I N I Y D I V K P A A A

A S R G P V V R L L D T R L I H H N E S K W E S F D V T P A I T R

W I A H K Q P N H G F V V E V T H L D N D T N V P K R H V R I S R S

L T L D K G H W P R I R P L L V T F S H D G K G H A L H K R Q K R

Q A R H K Q R K R L K S S C R R H P L Y V D F S D V G W N D W I V

A P P G Y H A F Y C H G E C P F P L A D H L N S T N H A I V Q T L V

N S V N T N I P K A C C V P T E L S A I S M L Y L D E N E K V V L

K N Y Q D M V V E G C G C R *

Fig. 4-2

(VII) BMP2B

M I P G N R M L M V I L L S Q V L L G G T N Y A S L I P D T G K

K K V A A D I Q G G G R R S P Q S N E L L R D F E V T L L Q M F G L

R K R P Q P S K D V V V P A Y M R D L Y R L Q S A E E E D E L H D

I S M E Y P E T P T S R A N T V R S F H H E E H L E N L P G T E E

N G N F R F V F N L S S I P E N E V I S S A E L R L Y R E Q I D H G

P A W D E G F H R I N I Y E V M K P I T A N G H M I N R L L D T R

V I H H N V T Q W E S F D V S P A I M R W T L D K Q I N H G L A I

E V I H L N Q T K T Y Q G K H V R I S R S L L P Q K D A D W S Q M R

P L L I T F S H D G R G H A L T R R S K R S P K Q Q R P R K K N K

H C R R H S L Y V D F S D V G W N D W I V A P P G Y Q A F Y C H G

D C P F P L A D H L N S T N H A I V Q T L V N S V N S S I P K A C C

V P T E L S A I S M L Y L D E Y D K V V L K N Y Q E M V V E G C G

C R *

Fig.4-3

(W) (Ver1)

M N A L T

V K R R L P V L L F L F H I S L S S I S S N T I L E N D F H S S F

V Q R R L K G H E R R E I Q K E I L T I L G L Q H R P R P Y L P E K

K K S A P L F M M D L Y N A V N I E E M H A E D V S Y S N K P I S

L N E A F S L A T D Q E N G F L A H A D T V M S F A N L V D N D N

E L H K N S Y R Q K F K F D L T D I P L G D E L T A A E F R I Y K D

Y V Q N N E T Y Q V T I Y Q V L K K Q A D K D P Y L F Q V D S R T

I W G T E K G W L T F D I T A T G N H W V M N P H Y N L G L Q L S

V E S M D M Q N V N P R L V G L V G K N G P Q D K Q P F M V A F F K

T S D I H L R S V R S T S N K H W N Q E R A K T Y K E Q D N L P P

A N I T D G I M P P G K R R F L K Q A C K K H E L F V S F R D L G

W Q D W I I A P E G Y A A Y Y C D G E C A F P L N S F M N A T N H A

I V Q T L V H F I N P E T V P K P C C A P T Q L N G I S V L Y F D

D S A N V I L K K Y K N M V V Q A C G C H *